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Ultrastructure of *Pseudomonas saccharophila* at Early and Late Log Phase of Growth

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The fine structure of *Pseudomonas saccharophila*, a soil bacterium, is similar to that of the marine *Pseudomonas* reported by Wiebe and Chapman. The unit membrane of the plasma membrane is clearly seen in some areas of thin sections. The ribonucleoprotein granules are distributed in the cytoplasm of the cell. Cells of *P. saccharophila* during early exponential phase are large, and most of them contain a large number of poly-β-hydroxybutyrate granules. Some of the granules are quite large and occupy up to three-fourths of the cross section of the cell. Thin sections of the cells in the late log phase, however, show fewer and smaller poly-β-hydroxybutyrate granules located in the central region of the cell. Negative-stained and freeze-fracture preparations show that the outer surface of the cell wall of *P. saccharophila* is covered with a large number of tiny granules and long, slender flagella. The outer surface of the plasma membrane appears to be smoother than the outer surface of the cell wall, and it also contains numerous granules. Since the outer surface of the cell wall is quite smooth in freeze-fracture preparations, the wrinkled appearance in thin sections is probably an artifact of fixation and dehydration. The poly-β-hydroxybutyrate did not solidify at the freezing temperature used (approximately −150 C), and it was consequently pulled out in a spikelike structure during the fracturing process. *P. saccharophila*, under the conditions in our study, appears to multiply by the constrictive type of cell division.

Since Kellenberger and Ryter developed a successful electron microscopy technique to study bacterial cells (8), the ultrastructure of numerous types of bacteria, the structural change under various environmental conditions or with the addition of different chemicals, and the relationship of structure and cell functions have been successfully examined by various electron microscopy techniques (5, 9, 17, 19-22). However, little has been reported on the alteration of fine structure at different stages of growth. This report describes the fine structure of *Pseudomonas saccharophila* at the early log phase and the late log phase of growth shown by electron microscopy with various techniques of preparation.

MATERIALS AND METHODS

Cultures. *P. saccharophila* was grown and maintained in a chemically defined growth medium, containing 0.1% NH_4Cl, 0.05% $MgSO_4$, 0.0005% $CaCl_2$, 0.005% ferric ammonium citrate, and 0.2% sucrose in 0.03 M sodium-potassium phosphate buffer, pH 6.8. A freshly grown 24-hr culture was used as the inoculum. Cells at the beginning and at the end of a log-phase culture (after 4 and 16 hr of growth in the medium, respectively) were used for electron microscope studies. The number of bacteria in the culture at early log phase was 20 to 22% of that at the late log phase. The increase of bacteria during the first 4 hr was equivalent to approximately 12% of the increase in 16 hr. The cells were then fixed immediately for electron microscope studies.

Negative stain technique. The cell suspension was mixed with 2% ammonium molybdate (1:1) for 10 min. A small droplet of this mixture was placed on a carbon-coated grid, and the excess fluid was removed by capillary action with filter paper. The cells were washed by placing a drop of 1% ammonium acetate on the grid, and then the excess ammonium acetate was removed by capillary action. The washing process was repeated twice to reduce the formation of ammonium molybdate crystals during vacuum-drying, which causes the ammonium acetate to sublime (7). Some negative-stained specimens were shadowed with uranium or platinum carbon at a 30° angle.

Freeze-fracture technique. Cell suspensions were fixed in 2.5% glutaraldehyde in phosphate buffer (18) for periods of 0.5 to 2.5 hr, centrifuged, and sus-

pended in 5% glycerine in phosphate buffer (*p*H 7.0) for 1 hr. This suspension was centrifuged, and the packed cells were loaded into copper mesh tubes rolled from 3-mm electron microscopy grids. The specimen tubes were then quickly frozen by immersion in liquid nitrogen-cooled Freon 22 and loaded into a Berkeley-etch Device (C. W. French Co., Weston, Mass.)

Electron microscopy techniques. The bacteria were initially fixed in freshly prepared 2% paraformaldehyde for 3 hr, as described by Peters and Ashley (14). The bacteria then were sedimented at 7,000 × *g* for 2 min and postfixed in 1% osmium tetroxide (OsO_4) overnight at 23 C by the method of Kellenberger and Ryter (8). After fixation, the bacteria were infiltrated and embedded in maraglas by a modified procedure of Freeman and Spurlock (6). The dehydration schedule was as follows: 50, 70, and 95% ethanol, then 100% ethanol three times, 5 min each; propylene oxide-maraglas (1:1), 3 hr; propylene oxide-maraglas (1:2), 3 hr; and maraglas, overnight. The embedding mixture consisted of 35 parts of maraglas, 9 parts of diepoxide flexibilizer, 5 parts of dibutyl phthalate, and 1 part of benzyldimethylamine. The bacteria were transferred to Beem polyethylene capsules filled with maraglas, and the capsules were hardened in an oven at 60 C. Ultrathin sections were cut with a diamond knife on a LKB microtome (no. 4800). Sections were floated on distilled water, collected on carbon- and collodion-coated 200-mesh stainless steel grids, and stained with lead citrate as described by Reynolds (16). Four to five grids were prepared on each culture. Approximately 12 fields were examined on each grid at a magnification of × 5,000 with a Hitachi electron microscope (HU-10) or a Phillips EM 300 electron microscope. At this magnification, each grid contained 50 to 100 bacteria. The electron micrograph was taken at ×10,000.

RESULTS

Fine structure of P. saccharophila in early log-phase culture. Cells of *P. saccharophila* used for this study were grown in a rotary incubator for 4 hr. Electron micrographs of thin sections show that the ribonucleoprotein particles (RNP) were rather uniformly distributed throughout the cytoplasmic region (Fig. 1A). Numerous areas that represent the location of the poly-β-hydroxybutyrate (PHB) were located along the central axis of the cell (Fig. 1A). The PHB granules were observed in many other bacteria by Pfister and Lundgren (15) and by Wang and Lundgren (20). The PHB areas were also observed in ammonium-starved cells in the presence of 0.2% sucrose, the so-called resting cells (not shown). PHB was found as the carbon storage and energy source for cellular metabolism such as protein synthesis. Our present results suggest that PHB formation precedes cell division. Occasionally, one or two electron-dense inclusion bodies were seen (Fig. 1A). The chemical nature of the inclusion bodies is not known. The nuclear region was not clearly observable in our electron micrographs but was considered to be in the central, lucent area similar to that shown in marine pseudomonads by Wiebe and Chapman (21). Numerous cells in the process of division were observed, and a mesosome-like structure was seen in association with the division process. The dividing cell showed a constricted area between the two new cells, but no transverse septum in the central region between two daughter cells has been observed (Fig. 1A).

The cross section of cells in the early log-phase culture showed that the PHB granules occupy about three-fourths of the cross section of the cell (Fig. 1B). A membranous structure surrounding the PHB granules was partially seen on the inner surface of some PHB granules. The membranous structure surrounding the PHB granules was previously reported by Wang and Lundgren (20).

The structure of the unit membrane was apparent in the plasma membrane of these cells, and certain areas of the outer surface of the cell wall also showed a double-membranous structure (Fig. 1B).

Fine structure of cell surface examined by negative stain and shadowed technique. The negative-stained cells, without shadowing, showed the areas of PHB granules and a few long, slender flagella (F) extending from the cell wall (CW) (Fig. 2). *P. saccharophila* cells were treated with ammonium molybdate, rinsed with water followed by ammonium acetate, and then shadowed with carbon platinum. The electron micrographs of cells after such treatment showed that the outer region of the cell flattened out, a result of the strong surface tension of water under vacuum (Fig. 3). The flattened area could be the cell wall that was dehydrated under vacuum drying. Long, slender flagella and different sizes and heights of the granules are also shown in Fig. 3.

Fine structure examined by freeze-fracture technique. The cell envelope and cytoplasm of *P. saccharophila* were also examined by the freeze-fracture technique. The electron micrographs show that the outer surface of the cell wall (CW) is covered with a large number of tiny granules of different sizes (Fig. 4). However, certain areas on the cell wall appear to be smooth and without granules (not shown). The outer surface of the plasma membrane (PM) was also covered with tiny granules. The PHB was not frozen at the freezing temperature used, approximately −150 C. Conse-

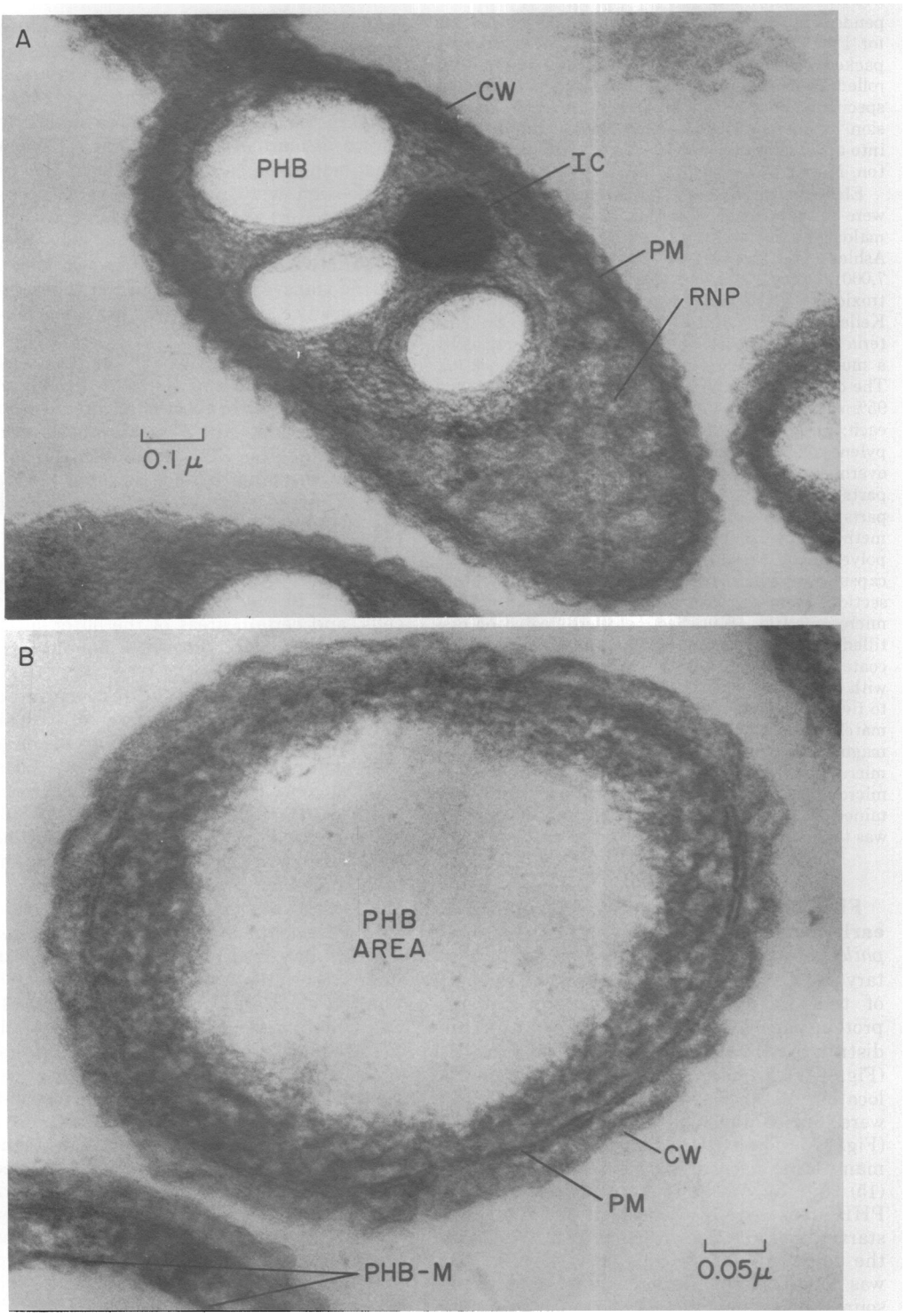

FIG. 1. *Thin sections of early log-phase P. saccharophila. Note large area of poly-β-hydroxybutyrate granules (PHB), ribonucleoprotein (RNP) granules scattered in the cytoplasm, inclusion body (IC), plasma membrane (PM), and cell wall (CW).*

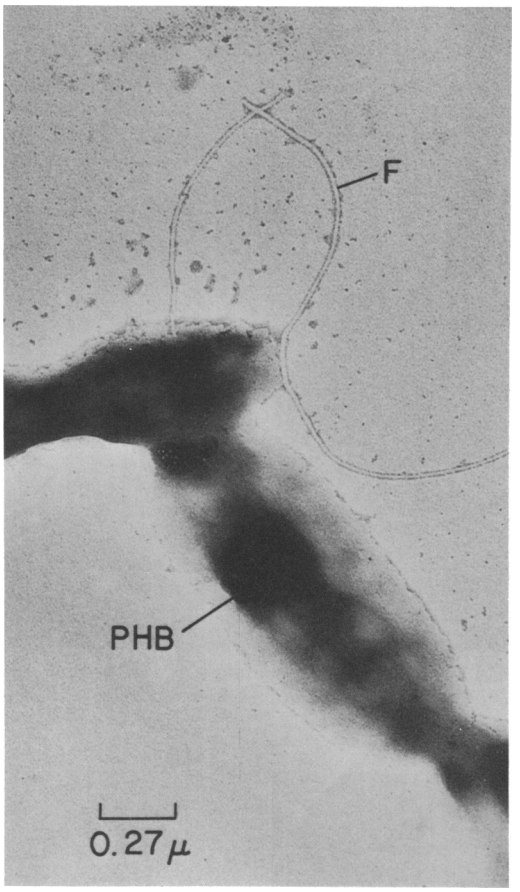

FIG. 2. *Electron micrograph of P. saccharophila negative-stained with ammonium molybdate without shadowing. It shows the PHB granules and the long, slender flagellum (F) on the bacterium.*

quently, it was pulled out in the form of a spike (S) during the fracturing process (Fig. 5). The spikelike structures were flexible and frequently moved during the shadowing, as shown by the double shadows in Fig. 5.

The PHB in early log-phase cells was extracted by the treatment of acetone after fixation. An attempt to fracture this extract was unsuccessful because it could not be frozen, and the extract was found to be quite pliable even after several hours of immersion in liquid nitrogen. In the cells from which PHB had been extracted, the original PHB areas appear to be empty, without spikes, and similar to the empty area shown in Fig. 5B, although this cell has not been extracted with acetone.

Fine structure of P. saccharophila in late log-phase cultures. Late log-phase cultures are the cultures grown for 16 hr at 30 C; the cells in such cultures enter their nondividing stage. The electron micrographs of thin sections of late log-phase cells show generally that these cells are smaller in comparison with the cells in early log phase. The cytoplasm of late log-phase cells is dense, and the ribonucleoprotein particles are rather uniformly distributed throughout the cytoplasm. There are a few small PHB granules present in some of the cells (Fig. 6).

DISCUSSION

Our results show that the fine structure of *P. saccharophila* is similar to the marine *Pseudomonas* reported by Wiebe and Chapman (21). In the early log phase, *P. saccharophila* accumulates large amounts of PHB in the form of granules (Fig. 1). These granules are located in the central region of the cell. Some of these granules are very large and occupy up to three-fourths of the cross section of the cell (Fig. 1B). The cells in the late log-phase cultures, however, contain fewer and smaller PHB granules than those in the early log-phase cultures. This suggests that, prior to cell growth and cell division, the cells accumulate nutrient in the form of PHB granules. In the late log-phase cultures, the nutrient is used up for cell divisions.

The thin sections of *P. saccharophila* show an undulant cell wall (Fig. 1) similar to that present in marine *Pseudomonas* reported by Wiebe and Chapman (21). However, the outer surface of the cell walls of *P. saccharophila* prepared by freeze-fracture techniques appears to be relatively smooth (Fig. 5). These results indicate that the undulating structure of the cell wall in thin sections probably is an artifact resulting from fixation and dehydration.

The electron micrographs of *P. saccharophila* prepared by freeze-fracture technique show that the outer surfaces of the cell wall and plasma membrane are covered with tiny granules (Fig. 4). The function of these granules is not known at present. Various enzymes (e.g., adenosine triphosphatase) are associated with and localized on various types of biological membranes such as plasma and mitochondrial membranes (2-4, 10-13). It would be of interest to investigate whether these tiny granules on the surface of the *P. saccharophila* are the site(s) of different enzymes. As mentioned above, certain areas on the cell wall appear to be smooth and without granules. Whether the granuleless area is the real structure of a living bacterium is not clear at

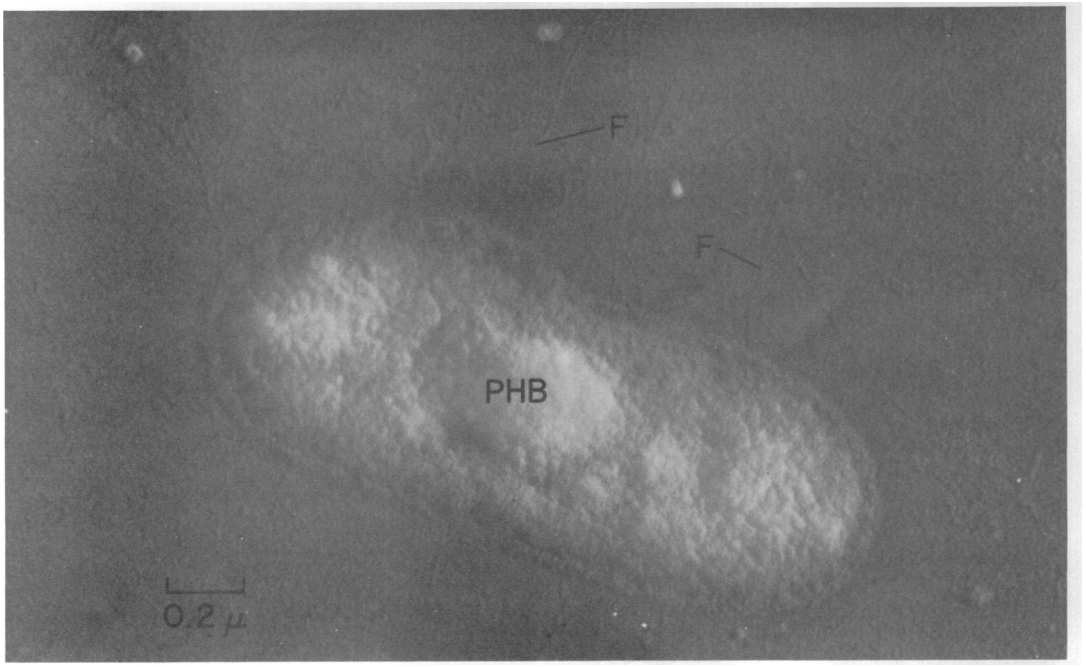

FIG. 3. *Electron micrograph of P. saccharophila treated with ammonium molybdate and then shadowed with carbon platinum. Note large PHB granules, long, slender flagellum (F), and granular surface of cell wall (CW).*

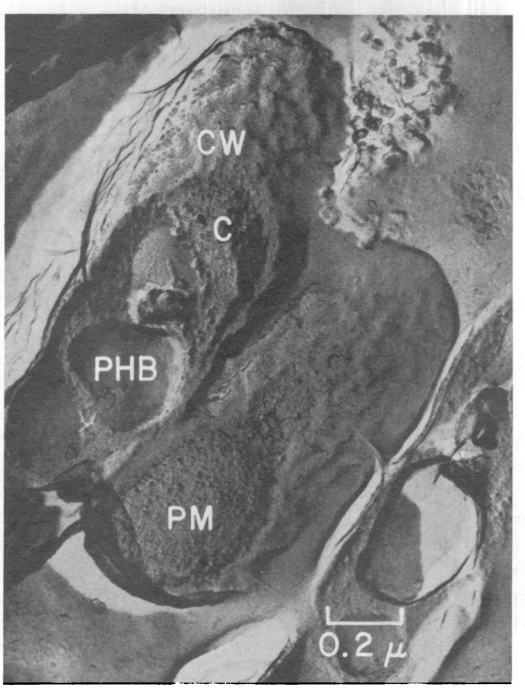

FIG. 4. *Electron micrograph of freeze-fractured P. saccharophila. Note granular structure of cell wall (CW) and plasma membrane (PM) and smoothness of inner surface of PHB granule.*

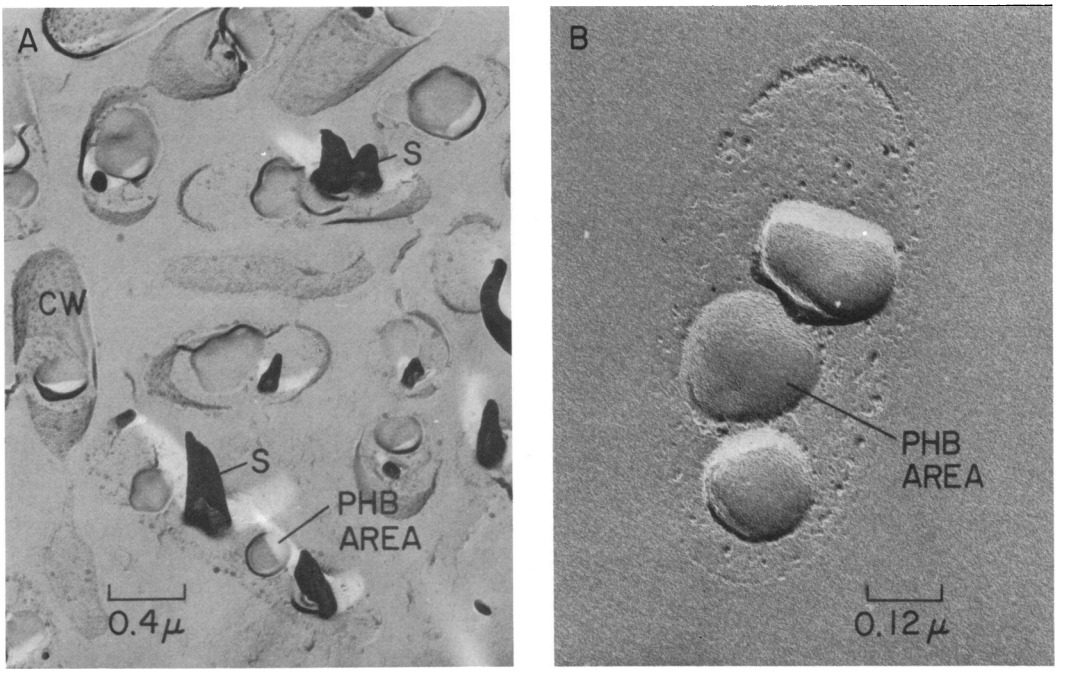

FIG. 5. *Electron micrographs of freeze-fractured P. saccharophila. (A) Note large spike structure (S) of PHB and double shadows of PHB which indicate the pliability of PHB at −150 C. (B) Note the rather smooth structure of the inner surface of PHB area.*

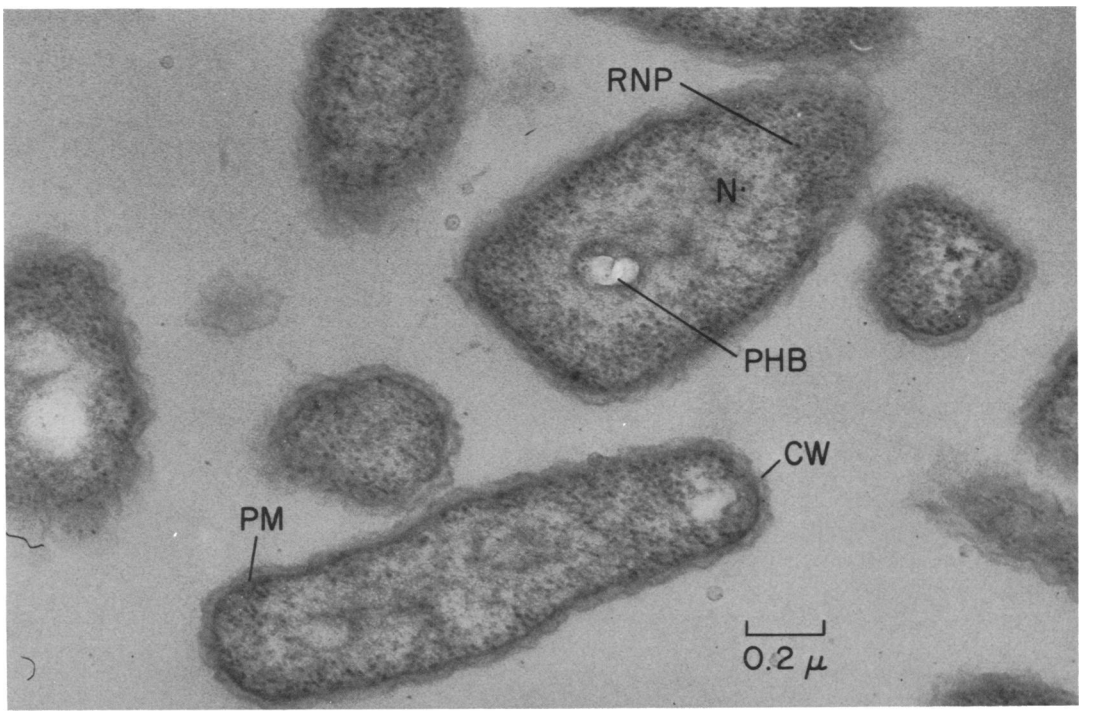

FIG. 6. *Thin section of late log-phase P. saccharophila. Note small size and number of PHB areas, dense cytoplasmic components, ribonucleoprotein granules (RNP), and lucent area of nucleus (N).*

present. It might be an artifact of specimen preparation or a shortcoming of the fracture technique. The latter may account for the inability to show the detailed cytoplasmic structure.

The electron micrographs of thin sections indicate that the cell wall between two dividing cells of *P. saccharophila* is formed by the extension and invagination of the original cell wall. Mesosome-like structures adjacent to the invagination were observed occasionally, and no transverse septum has been seen in the central region between the two daughter cells. The intracellular structures typical of mesosomal inclusions were observed recently also in eight strains of *P. aeruginosa* by Carrick and Berk (1). The observations reported here suggest that, under our experimental conditions, *P. saccharophila* multiplies by the method of constrictive division.

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